

Enhancement of Biological Nitrogen Fixation by Genetic
Manipulation of *Rhizobium* (PCM82-04730)

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Our efforts during the past year have been devoted to a continued investigation of the regulation of *nif* gene expression in *Rhizobium meliloti*, and to the study of other *Rhizobium* genes likely to be important in alfalfa symbiosis. A summary of this work is as follows:

(1) nif region:

We and others have previously characterized a 15 kilobase (kb) region of *E. meliloti* DNA which contains the nitrogenase genes (*nif* H, D, and K) and other genes necessary for symbiotic nitrogen fixation with alfalfa (Figure 1). The promoters, P1 and P2, control expression of a large portion of this *nif* gene cluster. Comparison of the DNA sequences of P1 and P2 has revealed that these two promoters share extensive sequence homology for more than 100 bp upstream from the RNA start site. It was therefore suggested (Better et al. (1984) Cell 35, 401) that this unusual promoter structure might be important in the coordinate regulation of P1 and P2 during symbiosis.

To determine how much of P1 or P2 DNA was essential for activity, we constructed a series of DNA deletion derivatives in which progressively more upstream DNA was removed. To monitor promoter activity, each of the deletions was fused to the *E. coli* β -galactosidase gene carried on a broad host range plasmid especially constructed for this purpose (see Section 5). After introduction into wild-type *E. meliloti* and inoculation of alfalfa, the β -galactosidase activity of nodule bacteroids was determined. Surprisingly, we found that only the first 30-35 bp preceding the RNA start was required for activity. This result was confirmed by introducing selected deletions into the genome of *Rhizobium* and determining that only deletions extending close to the transcriptional start site inhibit symbiotic nitrogen fixation.

The conclusion from such experiments is therefore that the conserved upstream regions of P1 and P2 are not required for symbiotic activation. Such a finding is surprising because of the following facts: (a) the region between -30 and the RNA start (+1) is highly conserved between *R. meliloti* and *K. pneumoniae* (Kpn), suggesting a commonality of function. In *Klebsiella*, evidence exists that the upstream regions of the *nif* H (analogous to P1) promoter and the *nif* LA promoter are required for activity. Furthermore, an *R. meliloti* gene exists with both structural and functional similarity to the Kpn transcriptional activator gene, *nif* A (Szeto et al., (1984) Cell 36, 1035). The *Rhizobium* gene, *Em nif A*, is within the *nif* gene cluster shown in Figure 1. (b) the upstream region, from -160 to -30, of P1 is functionally responsive to the Kpn *nif A* gene. Earlier work by Dr. F. Ausubel's group has shown that the Kpn *nif A* gene could activate P1 when the assay was performed in *E. coli*. We repeated this experiment with our deletion *nif* derivatives and found that the

upstream region of P1 was in fact essential for most of the observed activation.

A paper has been prepared summarizing these data, and a preprint will be sent as soon as available. In it, we offer several hypotheses to account for our observations. Probably the most intriguing idea is that the upstream region is actually involved in negative regulation of the promoter, maintaining it in an off configuration unless the proper plant host is present. Such a negative control might be essential to assure that R. meliloti is metabolically incapable of nitrogen fixation in the free living state (as seems to be the case), since a low nitrogen environment might otherwise cause the bacteria to futilely activate its nitrogenase genes. We are currently trying to test this idea by looking for physiological conditions (or by identifying mutants) where P1 deletions are active but the full P1 promoter is not. All in all, these experiments are telling us something fundamentally important about the way symbiotic bacteria like Rhizobium regulates its nif genes in comparison with free living nitrogen fixing bacteria like Klebsiella. Not unexpectedly, the situation is more complicated than a simple extrapolation might suggest.

A number of alternative approaches to studying transcriptional regulation via P1 and P2 have been devised and are summarized in our renewal proposal NSF PCM 82-04730, submitted 10/26/84.

Another aspect of transcriptional regulation which we have been investigating is the organization of expression downstream from P2. A detailed gene fusion analysis was undertaken employing the chloramphenicol acetyl transferase (CAT) gene cartridge on broad host range plasmids containing or lacking P2. We found that transcription from P2 terminates predominantly just prior to the Rm nif A gene (Figure 1), in agreement with earlier genetic data (Ruvkun et al. (1982) Cell 29, 551). On the other hand, we also found that 1/2 to 2/3 of the nif A expression in mature bacteroids is due to P2. Since nif A is a transcriptional regulator of P1 and P2, this finding may be of importance in determining the overall pattern of nif regulation.

A region whose expression has not yet been characterized is the 2 1/2 kb downstream from nif A. Fix genes in this region might be controlled by the nif A promoter, or they might be part of a separate operon. Preliminary evidence suggests that the latter may be the case. Fragments of DNA were sub-cloned from this region into a broad host range expression vector similar to that described earlier. Expression of β -galactosidase was found for one of these fragments and we are now attempting to verify the presumed RNA start site by S1 nuclease analysis and further sub-cloning. The DNA sequence of this promoter region would be of great interest, since we already know that it cannot have extended homology to either P1 or P2 (though limited homology, in the region between -30 and +1, might exist). The Rm nif A gene promoter, presently being characterized by Dr. Ausubel's laboratory, similarly does not have extensive homology to either P1 or P2.

The results of these experiments as well as the continued characterization of the P1/P2 promoters, are crucial to formulating a model for the control of expression of Rhizobium nif genes.

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(2) ALAS

Work has recently been completed on an analysis of the promoter-regulatory region of the γ -aminolevulinic acid synthetase gene of R. meliloti. This gene is responsible for the first step in heme biosynthesis and is most likely the controlling gene for the entire pathway. Evidence from the R. japonicum - soy system suggests that bacterial heme is a component of leghemoglobin.

Our laboratory was the first to identify and isolate δ -ALAS from a Rhizobium species (Leong et al. (1982) J. Biol. Chem. 257, 8724). Dr. Sally Leong, who was primarily responsible for this work, has since assumed a position with USDA at Madison, Wisconsin. In collaboration with Dr. Gary Ditta of our group, she has completed an analysis of the transcriptional regulation of this gene. A paper summarizing this work has just been completed and is in the final stages of readiness for publication.

We have found that the δ -ALAS gene is controlled by two different promoters located 250 base pairs apart. The possibility exists and has not been excluded that one or more small peptides encoded by the DNA between these promoters might encode a functional gene product. The DNA sequences of the two promoters are related and bear partial similarity to the consensus E. coli promoter sequence. No similarity exists to promoters known to be under general nitrogen control (e.g. nif). This result is of significance insofar as it is the first reported sequence for a presumptive Rhizobium "housekeeping" gene.

A genetic analysis, involving plasmid-borne fusions of various promoter deletions to E. coli β -galactosidase, revealed that both promoters function equally during vegetative and symbiotic growth. The relative activity of the δ -ALAS promoter is quite high, making it potentially useful for the constitutive expression of other genes in Rhizobium.

This project is the last major effort we will undertake with respect to δ -ALAS. We direct our available resources elsewhere. Some smaller scale investigations may, however, be carried out with the gene fusions that are already constructed.

(3) chromosomal "vir" genes

A major new effort in our laboratory has been initiated by the discovery of a gene in R. meliloti that is the functional equivalent of an Agrobacterium tumefaciens chromosomal (as opposed to Ti plasmid) vir gene. Based on DNA homology, we have identified a piece of Rhizobium DNA that can complement A. tumefaciens vir mutants for tumorigenicity. In Agrobacterium, defects in the chromosomal vir gene are characterized by a loss of the ability to bind to plant cells, in addition to being avirulent. This phenotype, coupled with the close taxonomic relatedness of R. meliloti and A. tumefaciens, makes the Rhizobium gene an important potential candidate for involvement in the early nodulation process. A preliminary mutant in the Rhizobium "vir" gene renders R. meliloti symbiotically defective. We are currently isolating additional mutants and characterizing the conservation of this gene in other Rhizobium species. A number of other experiments relating to the nature of this mutation in R. meliloti are planned. We feel this gene may be an extremely important key to further elucidating early nodule development by Rhizobium species.

(4) Rhizobium hormone genes

Many, if not most, soil bacteria seem capable of producing phytohormones. Rhizobium species have been characterized as producing both auxin and cytokinin. It is not known whether this capability plays an essential role in the symbiotic process. We therefore, undertook a number of preliminary studies to test the feasibility of comprehensively investigating indoleacetic acid (IAA; auxin) production by R. meliloti. The results of these studies were very promising and served as the basis for a research proposal recently submitted to the Department of Energy.

(5) development of plasmid vectors

We have continued to design and construct broad host range plasmid vectors for various phases of our work on Rhizobium. These vectors are of general utility to anyone working with gram-negative bacterias and we have correspondingly made them readily available to workers in related areas. A paper summarizing the various plasmids we have constructed and used during funding from NSF PCM 82-04730 has just been accepted for publication in Plasmid. A copy is enclosed.

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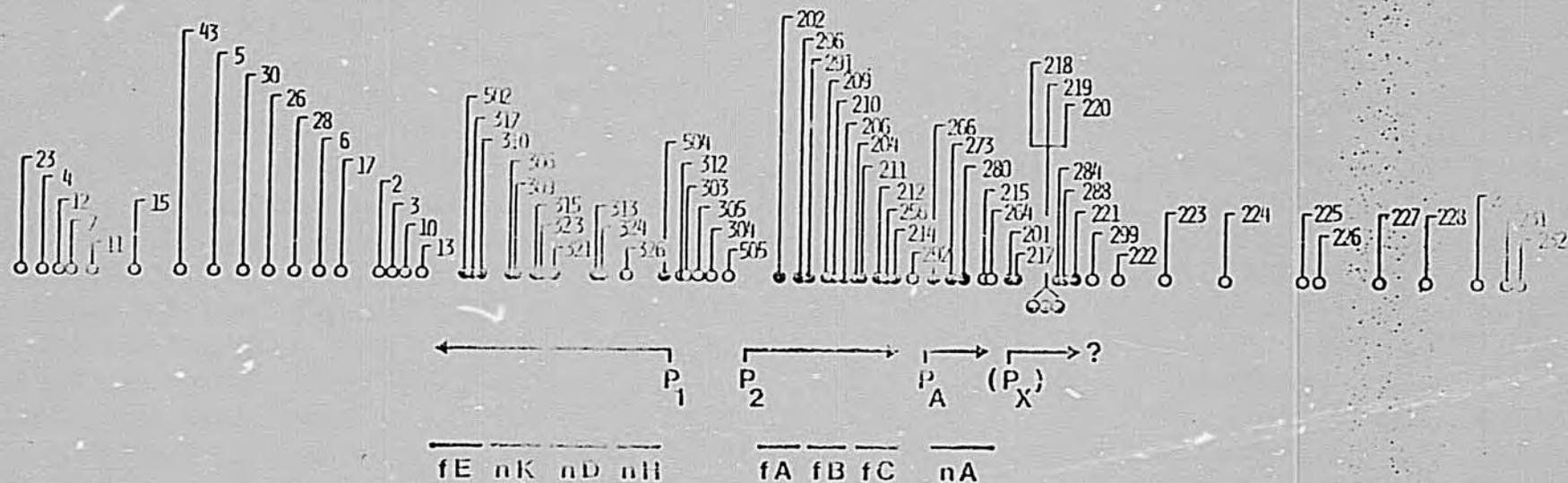


Figure 1

Nitrogenase region of *R. meliloti*. O, ● = fix^+ and fix^- transposon insertions defining symbiotically essential DNA. P₁ = nitrogenase promoter (22,32); P₂ = adjacent symbiotic promoter (22); P_A = nif A promoter (11); P_X = probable new promoter (see text). Arrows show direction and extent of transcription^X (tentative for P_A and P_X). f(A,B,C,E) = fix genes (75); n(H,D,K,A) = nif genes (11,21,76).